EX. 2

Biomed & Pharmacother 1999 ; 53 : 471-83

Original article

In-vivo delivery of therapeutic proteins by genetically-modified cells: comparison of organoids and human serum albumin alginate-coated beads

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Summary – We have designed a self-assembling multimeric soluble CD4 molecule by inserting the C-terminal fragment of the alpha chain of human C4-binding protein (C4bpα) at the C-terminal end of human soluble CD4 genes. This CD4-C4bpα fusion protein (sMulti-CD4) and two other reference molecules, a fusion protein of human serum albumin (HSA) and the first two domains of CD4 (HSA-CD4) and monomeric soluble CD4 (sMono-CD4), were delivered in vivo by genetically modified 293 cells. These cells were implanted in mice as organoids and also encapsulated in HSA alginate-coated beads. sMulti-CD4 showed an apparent molecular weight of about 300–350 kDa, in accordance with a possible heptamer formula. sMulti-CD4 produced either in cell culture or in vivo in mice appeared to be a better in-vitro inhibitor of HIV infection than sMono-CD4. Plasma levels of sMulti-CD4, HSA-CD4, and sMono-CD4 reached approximately 2,300, 2,700, and 170 ng/mL, respectively, 13 weeks after in-vivo organoid implantation, which had formed tumours at that time. This suggests that the plasma half-life of sMulti-CD4 is much longer than that of sMono-CD4. The 293 xenogeneic cells encapsulated in HSA alginate-coated beads remained alive and kept secreting sMono-CD4 or HSA-CD4 continuously at significant levels for 18 weeks in nude mice, without tumour formation. When implanted in immunocompetent Balb/c mice, they were rejected two to three weeks after implantation. In contrast, encapsulated BL4 hybridoma cells remained alive and kept secreting BL4 anti-CD4 mAb for at least four weeks in Balb/c mice. These results suggest the clinical potential of the C4bp-multimerizing system, which could improve both the biological activity and the poor in-vivo pharmacokinetic performance of a monomeric functional protein like soluble CD4. These data also show that a systemic delivery of therapeutic proteins, including immunoglobulins, can be obtained by the in-vivo implantation of engineered allogeneic cells encapsulated in HSA alginate-coated beads. © 1999 Éditions scientifiques et médicales Elsevier SAS

gene therapy / HIV / multivalent

Somatic gene therapy for delivering therapeutic proteins could be an attractive option for several clinical settings such as haemophilia B [1], growth hormone deficiency [2], mucopolysaccharidosis [3-5] or β thalassemia [6-11]. Current approaches are often based on the implantation of genetically modified autologous cells. Likewise, genetically modified fibroblasts can be prepared from a skin biopsy, expanded, and reimplanted after their aggregation on fibres. Such 'organoids' become vascularized and secrete the recombinant proteins of interest. However, such a 'tailored' therapy, in which the genetic modification as well as the necessary quality control tests need to be performed for each patient, is labour-intensive and costly. It might therefore be applicable for the treatment of rare diseases, but not for prevalent diseases such as HIV infection. For the latter settings, the design of a strategy that allows the implantation of nonautologous cells is an alternative approach that is currently under investigation. Indeed, new technologies such as the encapsulation of allogeneic cells, are under development [12]. They are aimed at protecting the transplanted cells from being rejected by the host immune system while allowing the secretion of the therapeutic proteins [13, 14].

We aim to develop a new therapy for HIV infection based on the secretion of antiviral proteins. We previously reported the long-term in-vivo delivery of a soluble form of the HIV receptor, the CD4 molecule. Mouse fibroblasts were retrovirally transduced with a soluble CD4 gene (sCD4), and reimplanted as organoids in transgenic mice expressing human CD4 and thus tolerant to sCD4 [15]. The sCD4 serum levels obtained were significant but too limited for an

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expected antiviral effect. This appears primarily due to the pharmacokinetic properties of sCD4, which has a very short plasma half-life [16]. We and others have designed CD4-based recombinant chimeric proteins aimed at increasing their anti-HIV properties as well as their half-lives [17-21]. CD4-Ig molecules are still under development for clinical use, in association with other antiviral molecules such as reverse transcriptase inhibitors or anti-proteases. We have already designed a fusion protein comprising human serum albumin (HSA) and the first two domains of CD4 (HSA-CD4) [20]. This recombinant protein has a long half-life in the serum, comparable to that of human serum albumin. We also recently designed a multimeric CD4 fusion protein based on a C-terminal fragment of human C4 binding protein alpha chain (C4bpα), a naturally heptameric protein [22]. CD4-C4bpα should thus be secreted as a heptamer, and such a large and multimeric molecule is expected to have longer half-life, better stability in vivo, and better anti-HIV activity.

We thus aimed to analyse the feasibility of secreting anti-HIV therapeutic proteins by organoids or cell encapsulation into HSA alginate-coated beads. We generated stable cell lines secreting either sMono-CD4, HSA-CD4 or sMulti-CD4. We then reimplanted these cells as organoids or after encapsulation in HSA alginate-coated beads [23], and monitored the expression of the recombinant proteins in sera of immunodeficient or immunocompetent mice. Our results indicate that plasma concentrations dramatically depend on the number of secreting cells and also on the half-life of the recombinant proteins. Plasma concentrations of HSA-CD4 up to 125 μg/mL were achieved. Furthermore, we show that allogeneic cells encapsulated in the coated alginate beads could represent an efficient way to secrete recombinant therapeutic proteins.

These results warrant further developments of this therapeutic strategy for the treatment of HIV infection. Indeed, CD4-based molecules, neutralising antibodies directed against viruses or against HIV receptors or co-receptors [24], are capable of inhibiting HIV infection and could benefit from these delivery methods.

MATERIALS AND METHODS

Construction of expression plasmids

The 177-base pair C-terminal C4b*P* α-chain fragment was amplified using the following primers:

5'-GAGACCCCCGAAGGCTGTGA-3'

5'-ATTTCTAGAGAGTTATAGTTCTTTATCCAAAG TGGA-3'

The underlined sequence represents the XbaI restriction site. The stop codon is shown in bold characters. The polymerase chain reaction was accomplished as previously described, by using genomic DNA of HepG2 cells as a template [22].

The 6.2 kb pST4 plasmid containing the sequence of the four extracellular domains of human CD4 [25] was digested by EcoRI and AvaI. CD4 coding fragment was linked at its 3' end to an AvaI-digested oligonucleotide:

(5'-**TCGGA**ACAGGTCCTGCTGGAATCCAACAT-CAAGGTTCTGCCCACATGG-3') previously linked to the C4b*P* α-chain fragment. This EcoRI-XbaI fragment was then subcloned into the multiple cloning site of pCI plasmid (Promega) to make CD4-C4bpα/pCI. This construct led to the expression of a multimeric covalent molecule referred to as sMulti-CD4.

A coding fragment of sMono-CD4 from pM48-sCD4 [15] or that of HSA-CD4 from pYG365B [20], was also cloned into the multiple cloning site of pCI to make sCD4/pCI and HSA-CD4/pCI, respectively *(figure 1)*.

Selection of sMulti-CD4, HSA-CD4 or sMono-CD4 secreting 293 clones

Subconfluent 293 cells (ATCC CRL 1573) in a 10 cm dish, which were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were co-transfected with 0.5 μg of pMC1neo poly A (Stratagene) and 20 μg of CD4-C4bpα/pCI, HSA-CD4/pCI or sCD4/pCI by standard calcium phosphate co-precipitation method. Forty-eight hours later, G418 was added to the medium at 500 μg/mL. After 14 days, neomycin-resistant clones were isolated and individually expanded. Secretion of the recombinant protein was then assayed by enzyme-linked immunosorbent assay (ELISA).

ELISA procedures

For HSA-CD4, ELISA plates (Maxisorp, Nunc, Denmark) were coated with IgG fraction of a polyclonal rabbit anti-HSA serum (Sigma, dilution 1:2000), blocked with 1 x Power Block® solution (BioGenex, USA), and incubated with samples of culture supernatant or plasma from the organoids-implanted mice, which were diluted with 1 x Power Block® solution. Leu3a anti-CD4 mAb or Leu3a conjugated with biotin (Becton and Dickinson, dilution 1:125) was then added, followed by the peroxidase-linked anti-mouse IgG serum (DAKOPATTS, dilution 1:2000) or streptavidin-POD (Boehringer Mannheim, dilution 1:800). Absorbance at 405 nm was measured after the addition of ABTS solution (Boehringer Mannheim).

All sMulti-CD4 and sMono-CD4 measurements in culture medium and plasma were performed using the sCD4 ELISA kit (Boehringer Mannheim) to detect the number of CD4 binding sites for the revealing antibody. Results are thus expressed in ng/mL with respect to a standard curve made with purified recombinant sCD4.

Protein electrophoresis and Western Blotting

Electrophoresis was performed in a precast Tris-Glycine 4–12% polyacrylamide gel (Novex, USA). Pre-stained Rainbow markers (Amersham, UK) were used for gel calibration. The culture supernatant of the 293 cells secreting sMulti-CD4, HSA-CD4 or sMono-CD4, or diluted plasma of the organoid-implanted nude mice were loaded in reducing (Tris-HCl 63 mM, Glycerol 10%, SDS 2%, dithiothreitol [DTT] 50 mM) or non-reducing condition (Tris-HCl 63 mM, Glycerol 10%, SDS 2%). After electrophoresis, proteins were transferred to a nitrocellulose membrane (0.45 µm pore size, Shleicher & Schuell) at 4° C overnight by electro-transfer using Mini-TransBlot (BioRad). To ensure equivalent protein loading in each lane and correct transfer, bands were made visible by staining with 0.3% Ponceau Red staining. After staining, membranes were rinsed in TBS. The filter was blocked, incubated with a rabbit polyclonal anti-human CD4 serum (K582) [19] for sMulti-CD4 and sMono-CD4, or with a rabbit polyclonal anti-HSA IgG (Sigma) for HSA-CD4, then developed using peroxidase-linked anti-rabbit IgG serum (DAKOPATTS) and a chemiluminescence Western Blot kit (Boehringer Mannheim).

HIV-1 viruses and viral infection procedures

P4-CCR5 infection: The derivation and characterization of the P4-CCR5 cells has been previously described [26]. Cells are grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco), 500 µg/mL G418 and 1 µg/mL puromycin. The P4-CCR5 cells were plated (10^4 cells/well of 96-well plate, 3×10^4 cells/well of 48-well plate) one day prior to infection. On the day of the infection, 100 tissue culture infectious doses of HIV-1_{LAI} strain of HIV-1 (HIV-1_{LAI}, Diagnostics Pasteur, France) were first incubated with several concentrations of sMulti-CD4 or sMono-CD4 for two hours. Then those mixtures of HIV-1_{LAI} and sMulti-CD4 or sMono-CD4 are added to the cells for infection in the presence of 20 µg/mL of DEAE Dextran. After 48 hours of infection, the medium was removed and the enzymatic detection of β-Gal was performed using a β-gal reporter gene assay kit (Boehringer Mannheim).

Organoid formation and implantation

The 293 clones secreting sMulti-CD4, HSA-CD4 or sMono-CD4, or naive 293 cells were used to make organoids. Organoids were surgically implanted into the peritoneal cavity of six-week male Swiss (nu/nu) nude mice (Iffa-Credo, France) anaesthetised with Tribromoethanol (Acros Organics, New Jersey, USA) at 125 mg/kg. Neo-organ formation, implantation, and removal were performed as previously described [15].

All animal studies were performed at the animal facility of the Centre d'Étude et de Recherche Viro-Immunologiques (CERVI), Hôpital de la Pitié-Salpêtrière, Paris, France. The animals were cared for in accordance with local institutional guidelines.

Preparation of the human serum albumin alginate-coated beads and bio-encapsulation of cells

The procedure was as described [23] with minor modifications. The initial aqueous phase was prepared by dissolving three components, sodium alginate (1.25%), propylene glycerol alginate (PGA, 2.5%) and human serum albumin (HSA, 6.25%) in 0.9% NaCl. After 5 min of stirring, the homogeneous solution was centrifuged for 5 min at 5,000 rpm to eliminate the air bubbles. Twenty-eight million cells were collected in 500 µl of sterile 0.9% NaCl, then carefully added and re-suspended in 2 mL of the alginate solution. The resulting solution containing cells was added dropwise to 12.5 mL of 10% $CaCl_2$ to form beads. After 5 min of magnetic stirring, the beads were washed with 0.9% NaCl, then the transacylation reaction between the ester groups of PGA and the amine group of HSA was initiated in 10 mM NaOH for 5 min under agitation. A membrane was formed around the beads, made of a protein directly bound to polysaccharides through amide linkages. The coated beads were then incubated for 5 min in 0.1 M Imidazol buffer, pH 7.0, then incubated in cell culture medium. One batch of beads corresponds to approximately 140 beads. In this condition, the cell number in beads, at the beginning of the culture, is theoretically about 200,000 cells/bead.

Mice plasma analysis: transgene expression in vivo in Swiss nude mice

Retro-orbital blood was collected from the organoid- or bead-implanted mice with a 100 µL heparinated capillary pipette under anaesthesia with Tribromoethanol. The collected blood was centrifuged at 5,000 rpm (2,700 relative centrifugal force) and the plasma was immediately stored at –20° C. sMulti-CD4, HSA-CD4 or sMono-CD4

were analysed by ELISA and immunoblot on thawed aliquots.

Analysis of BL4 anti-CD4 mAb secretion

BL4 (Immunotech, Marseilles, France: Hybridoma Data Bank, BL4 195 RE 1.G) is a monoclonal antibody which specifically reacts with human CD4 [27]. The BL4 hybridoma cells were produced by fusion of the mouse myeloma Sp2/o with spleen lymphocytes from a BALB/c mouse immunized with human peripheral blood T lymphocytes. A monoclonal antibody (IgG2a κ), BL4, is produced.

Sera were collected from the mice that were implanted with the encapsulated BL4 hybridoma cells and analyzed for the presence of BL4 anti-CD4 mAb. Two million human peripheral blood lymphocytes (PBLs) were incubated with 50 µL of different serum at 1:5 or 1:20 in PBS-1% BSA for 30 min at 4° C. After three washings, PBLs were then incubated with 50 µL of anti-mouse-FITC antibody at 1:50 in PBS-1% BSA for 30 min at 4° C. After three washings, PBLs were analysed on a FACStar Plus apparatus (Becton Dickinson).

Histologic analysis of the human serum albumin alginate-coated beads

The cells containing human serum albumin alginate-coated beads were recovered from the mice, washed with PBS and fixed with PBS containing 2% glutaraldehyde for 48 h. Beads were then dehydrated in 70, 80, 90, 95 and 100° ethanol successively and put in propylene oxide (1 h for each stage). Beads were then incubated in 50% propylene oxide/50% EPON (Merck) for 2 h, and in EPON overnight. Finally, the beads were put in EPON solution with 1.5% of the catalyser (2,4,6-Tris[Dimethylaminomethyl]Phenol: DMP30) DMP30 for 72 to 96 h at 60° C to allow the resin to polymerize. Finally, samples were cut using an ultramicrotome and stained with 0.5% methylene blue (Sigma) and 0.5% azur blue II (Merck) for observation.

RESULTS

In-vitro expression and characterization of sMulti-CD4, HSA-CD4 and sMono-CD4 by 293 cells

Neomycin-resistant clones of 293 cells transfected with CD4-based expression vector together with pMC1neo poly A were isolated by G418 selection. They were grown to confluence, fed with fresh medium without FCS, and 24-hour culture supernatants were collected and assayed for the recombinant protein secretion by ELISA. We selected clones secreting sMulti-CD4, HSA-CD4 or sMono-CD4 at a rate of, 4.0, 1.1 and 4.2 µg/10^7 cells/24 hours, respectively.

To verify the nature of these secreted proteins, Western Immunoblot analysis was performed using a polyclonal anti-hCD4 rabbit serum [19] *(figure 1C)*. Unique 44 or 92 kDa bands were detected under both reducing or non-reducing conditions with the supernatant of the 293/sCD4 (lanes 2, 6) or 293/HSACD4 (lane 10), respectively. The 92 kDa band could also be detected using a polyclonal anti-HSA serum (lane 12). A unique 50 kDa band corresponding to the molecular weight of monomeric CD4-C4bpα could also be seen under reducing condition with the supernatant of 293/CD4-C4bpα cells (lane 1). Under non-reducing condition, a unique band of large molecular weight (≈350 kDa) could be detected (lane 5). These results indicate that the CD4-based recombinant proteins are efficiently secreted by 293 cells as unique soluble products, probably heptameric CD4-C4bpα.

sMulti-CD4 inhibits HIV infection better than sMono-CD4

To determine whether sMulti-CD4 secreted by 293 cells retains anti-HIV infection activity, culture supernatant of 293/CD4-C4bp was tested in HIV infection of P4-CCR5 cells in vitro. Several dilutions of the culture supernatant of 293/CI/CD4-C4bp or 293/CI/sCD4 were incubated with 100 tissue culture infectious doses (TCID) of HIV-1_{LAI} for 48 h. The 50% inhibitory concentration of sMulti-CD4 was 35 ng/mL, three times better than that of purified recombinant soluble CD4, 100 ng/mL *(figure 2)*.

These results indicate that the sMulti-CD4 and sMono-CD4 protein produced by the 293 cells are functional. On the contrary, the HSA-CD4 recombinant protein showed a poor capacity to block HIV infection (data not shown).

In-vivo delivery of sMulti-CD4, HSA-CD4 and sMono-CD4 by organoids implanted in nude mice

Using the 293/CD4-C4bp, /HSA-CD4 or /sCD4 cells, we generated the organoids made of 107 cells. Two organoids were transplanted into the peritoneal cavity of each Swiss nude mouse.

Implantation of sMulti-CD4-secreting organoids resulted in high plasma levels of sMulti-CD4, which increased from 5.5 ± 1.4 ng/mL (mean ± SEM) at two

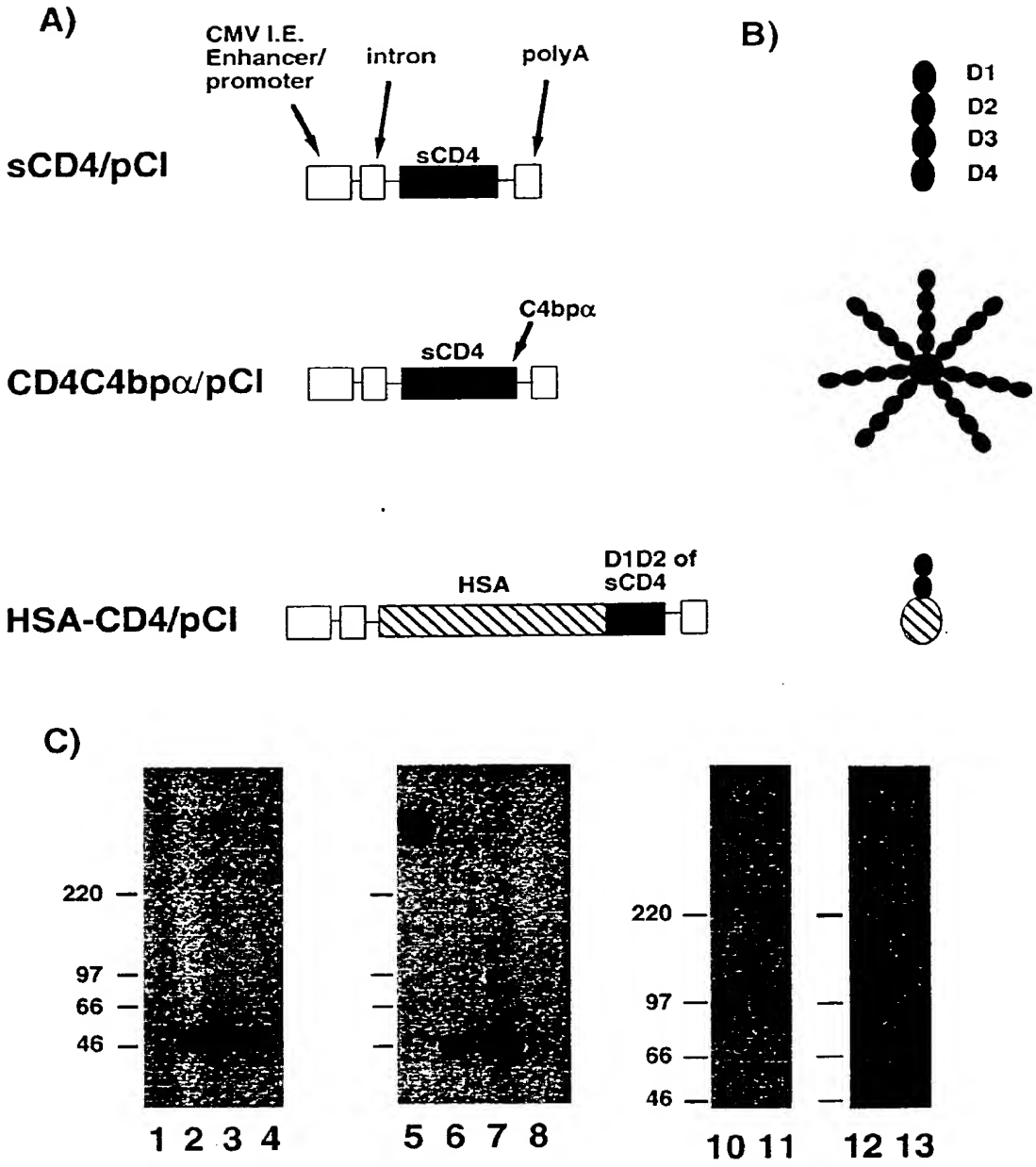

Figure 1. Expression in 293 cells. A) Expression plasmids: pCI/sCD4, pCI/HSA-CD4 and pCI/CD4-C4bpα plasmids coding for human sMono-CD4, HSA-CD4 and sMulti-CD4, respectively. B) Schematic presentation of CD4-based proteins. sCD4 consists of four domains. CD4-C4bpα is a hybrid gene resulting in the association between sCD4 and C4bpα genes that allows the secretion of sCD4 homo-multimeric molecules. HSA-CD4 is a hybrid of human serum albumin (HSA) merged to the first two domains of human CD4. C) Expression of sMulti-CD4, sMono-CD4, and HSA-CD4 by 293 cells in vitro. Immunoblot analysis of culture supernatants, as revealed by a polyclonal serum directed against human CD4 under reducing (lanes 1–4), non-reducing (lanes 5–8); supernatants of the 293 cells secreting sMulti-CD4 (lanes 1, 5), and those of soluble CD4 (lanes 2 and 6); diluted purified soluble CD4 (lanes 3 and 7) as positive control; supernatants of untransfected 293 cells (lanes 4 and 8). Polyclonal serum against human serum albumin (lanes 10 and 11) and polyclonal serum directed against human CD4 (lanes 12 and 13) revealed 92 kDa bands corresponding to HSA-CD4 in the supernatants of 293/CI/HSA-CD4 (lanes 10 and 12); supernatants of untransfected 293 cells (lanes 11 and 13).

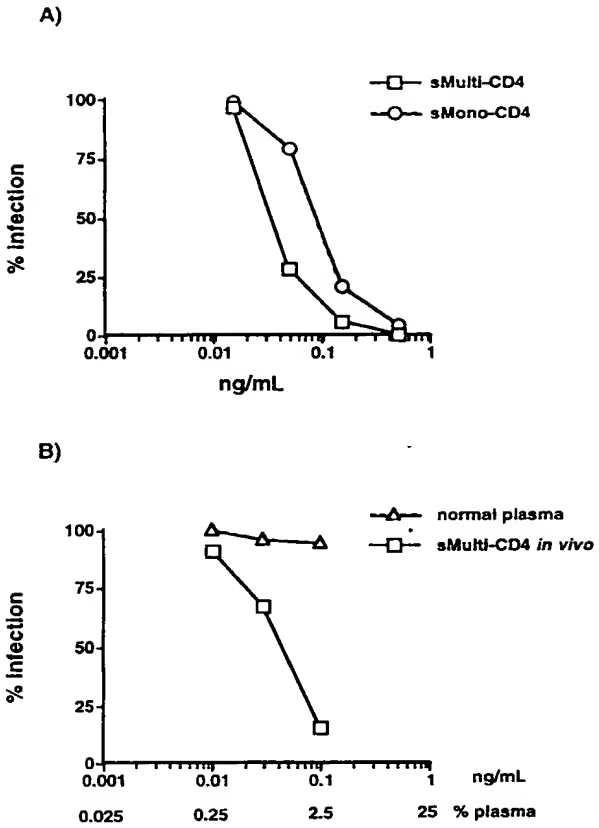

Figure 2. Antiviral activity of sMulti-CD4. **A)** sMulti-CD4 by 293 cells in vitro: P4-CCR5 indicator cells were infected with 100 tissue culture infectious doses of HIV_{LAI} with different concentrations of sMulti-CD4 or sMono-CD4 secreted by 293 cells in vitro for 48 h, then enzymatic detection of β-gal was performed. Results represent the percentage of the β-gal compared with that of β-gal of P4-CCR5 cells infected with HIV_{LAI} without any sMono-CD4 or its derivatives. **B)** sMulti-CD4 expressed in vivo: P4-CCR5 cells were infected with HIV_{LAI} with different dilutions of the plasma of the nude mouse implanted with 293/CI/CD4-C4bpα-organoids. As a negative control, we infected the P4-CCR5 cells with HIV_{LAI} in the presence of the supernatant of naive 293 cells and it showed no significant effect on HIV infection at less than 2.5%. But at more than 7.5%, normal plasma also showed antiviral activity, as those of Multi-CD4 or sCD4.

weeks post-implantation to 2,306 ± 716.7 ng/mL, when they were sacrificed. With 293/HSA-CD4-organoids, the HSA-CD4 plasma concentrations were 2.6 ± 0.8 ng/mL at two weeks post-implantation and raised to 5,432 ± 258 ng/mL at 13 weeks post-implantation. In contrast, we could not detect any plasma sMono-CD4 at two weeks post-implantation in 293/sCD4 organoid-implanted mice, and only 1.1 ± 0.5 ng/mL and up to 173 ± 91.6 ng/mL at four and 13 weeks post-implantation, respectively *(figure 3A)*.

Starting at four to five weeks after transplantation, all nude mice developed tumours which continued to grow until they were sacrificed for examination. All the neo-organs were found to be connected to the mesenteric tissues or to the bowel, and were well vascularized. Organoid weights, which were approximately 0.2 g before transplantation, rose to 11.4 ± 5.28 g (mean ± SD, $n = 5$) with 293/CD4C4bpα organoids and 17.53 ± 1.60 g ($n = 3$) for 293/HSA-CD4 (not significantly different), whereas that of 293/sCD4 were significantly smaller (2.44 ± 2.85, $n = 5$, paired T test, $p < 0.05$). No signs of metastatic tumours were seen at macroscopic evaluation. Thus, 293 cells are tumourigenic in nude mice.

Characterization of in vivo-expressed recombinant proteins

To investigate if the in vivo-expressed sMulti-CD4 was still multimeric, we performed Western Immunoblot with the plasma of a 293/CD4-C4bpα organoid-implanted mice *(figure 3B)*. The polyclonal anti-hCD4 serum showed a unique high molecular band (lane 5) identical to that observed with the culture supernatant of these same cells (lane 2), and thus corresponding to sMulti-CD4 molecules.

Plasma from HSA-CD4-organoid-implanted mice was also examined using Western Blotting. A polyclonal anti-HSA serum revealed the 92 kDa band corresponding to the HSA-CD4 hybrid (lane 6). The polyclonal antibodies (either anti-hCD4 or anti-HSA) also reacted non-specifically with plasma proteins, resulting in a heterogeneous band pattern identical between organoid-implanted (lanes 5, 6) and control mice (lanes 4, 7).

Relationship between plasma levels and numbers of secreting cells

Total body weight plotted versus the plasma concentration at various time points showed a good linear correlation *(figure 3C)*, suggesting that the 293 cells kept secreting recombinant proteins during tumour growth. It also indicates that the plasma concentration is a linear function of the number of secreting cells.

When the animals were sacrificed, implants were removed, weighed, and the cells were dissociated with collagenase. They were then cultured in vitro with or without G418, and analysed for the secretion of the transgene products. The recovered 293/CD4-C4bpα

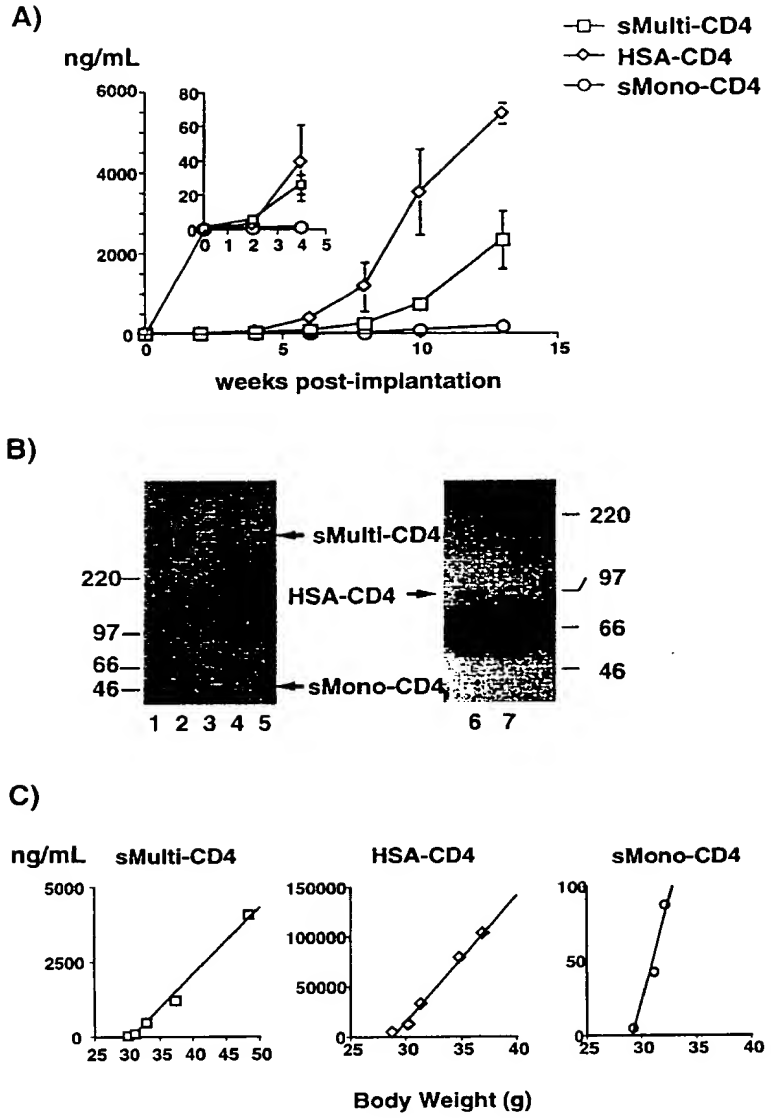

Figure 3. In-vivo expression of sMulti-CD4, HSA-CD4 and sMono-CD4 by organoids in nude mice. A) Plasma level of sMulti-CD4, HSA-CD4 and sMono-CD4 as a function of time after implantation of the organoids. Swiss nude mice were implanted either with the 293/CI/CD4-C4bpα-organoids (n = 6), the 293/CI/HSA-CD4-organoids (n = 3) or the 293/CI/sCD4-organoids (n = 6), then bled at the indicated number of weeks after the implantation; the plasma was stored frozen. The expression of those molecules was determined by ELISA and is presented as mean ± SEM. B) Immunoblot analysis of the plasma using a polyclonal serum directed against human CD4 under non-reducing condition: lane 1, molecular weight marker; lane 2, supernatants of 293 cells secreting sMulti-CD4; lane 3, supernatants of the 293 cells secreting sCD4; lane 4, 1:2 diluted plasma from untreated Swiss nude mouse; lane 5, 1:2 diluted plasma from a 293/CI/CD4-C4bpα-organoids-implanted nude mouse. Immunoblot with a polyclonal rabbit against HSA under reducing condition in lanes 6 and 7. Lane 6, 1:50 diluted plasma from a 293/CI/HSA-CD4-organoid-implanted nude mouse; lane 7, 1:50 diluted plasma from an untreated Swiss nude mouse. C) Significant correlation between body weight (BW) and the plasma concentration: BW and plasma concentration of sMulti-CD4,HSA-CD4 or sMono-CD4 in organoid-implanted mice at each time point were plotted. Significant correlation between them was shown (sMulti-CD4, $r^2 = 0{,}989$; HSA-CD4, $r^2 = 0{,}991$; sMono-CD4, $r^2 = 0{,}946$). Data shown are from a representative animal of each experimental group.

cells were secreting sMulti-CD4 at the same level with or without G418, 22% lower than their secretion level before implantation. In contrast, the recovered 293/HSA-CD4 or 293/sCD4 cells retained the secretion capacity they showed before implantation.

Plasma sMulti-CD4 retains its capacity to inhibit HIV infection

We next investigated whether sMulti-CD4 expressed in vivo in nude mice retains its anti-HIV activity, with an HIV inhibition assay based on using P4-CCR5 cells [26]. These Hela-derived cells co-express human CD4 and CXCR5 and are thus sensitive to HIV infection. They are also transduced with a Tat-inducible Lac-Z gene in order to monitor the HIV infection by Lac-Z expression. The P4-CCR5 cells were infected with 100 TCID of HIV-1_{LAI}, in the presence of different dilutions of either the plasma from 293/CD4-C4bp-organoid-implanted mice which contains 4 ug/mL of sMulti-CD4, or the plasma of normal nude mice as a negative control *(figure 3)*. After 48 h, the enzymatic

detection of β-Gal was performed. The results indicated that in-vivo-expressed plasma sMulti-CD4 retained an anti-HIV activity similar to that from in-vitro expressed sMulti-CD4.

In-vitro secretion of therapeutic proteins from encapsulated 293 cells

We investigated the secretion of the recombinant proteins after the encapsulation of 293 cells in human serum albumin alginate-coated beads. Encapsulated 293 cells were placed in culture medium, and the secretion rate of sMono-CD4, HSA-CD4 or sMulti-CD4 was analysed by ELISA. At four weeks after encapsulation, secretion rates were 3,307, 751 and 3 ng/30beads/d, respectively. Previous analysis allowed us to determine that 30 beads contain approximately 6 x 10^6 cells. The three 293 clones secrete sMono-CD4 (44 kDa), HSA-CD4 (92 kDa) and sMulti-CD4 (350 kDa) at 2,372, 663 and 2,452 ng/24 h/6 x 10^6 cells, respectively. Thus, sMono-CD4 and HSA-CD4 freely diffused through the beads, but sMulti-CD4 didn't.

Xeno-transplantation in immunocompromised mice: in-vivo expression of sMono-CD4 and HSA-CD4 from the encapsulated 293 cells in Swiss nude mice

In order to evaluate if the encapsulated xenogeneic 293 cells could secrete sMono-CD4 or HSA-CD4 without tumour formation, 30 beads with 293/sCD4 or 293/HSA-CD4 cells were implanted into each mouse and the plasma concentration of sMono-CD4 and HSA-CD4 measured over time. The HSA-CD4 plasma concentration reached a plateau of ≈300 ng/mL at four weeks post-implantation *(figure 4A, upper panel)*, and stayed at this level for more than 16 weeks post-implantation. Plasma sMono-CD4 concentration was 0.5 ng/mL two weeks post-implantation and remained at the same level during 13 weeks *(figure 4A, lower panel)*.

The HSA alginate-coated bead-implanted mice were sacrificed and examined macroscopically. All beads were found intact in the peritoneal cavity and easily recovered. There was no sign of tumour growth of the cells outside the beads. In contrast, the identical 293 clones implanted as organoids in nude mice showed tumour formation in all the mice after four to six weeks of implantation. Beads were recovered, put in culture medium in vitro, and analysed for transgene secretion. The recovered 293/sCD4 and 293HSA-CD4 cells in the beads were shown to keep secreting their transgene products at 4,000 and 400–880 ng/30 beads/24 h respectively after their recovery, which is about the same rate as those of before implantation.

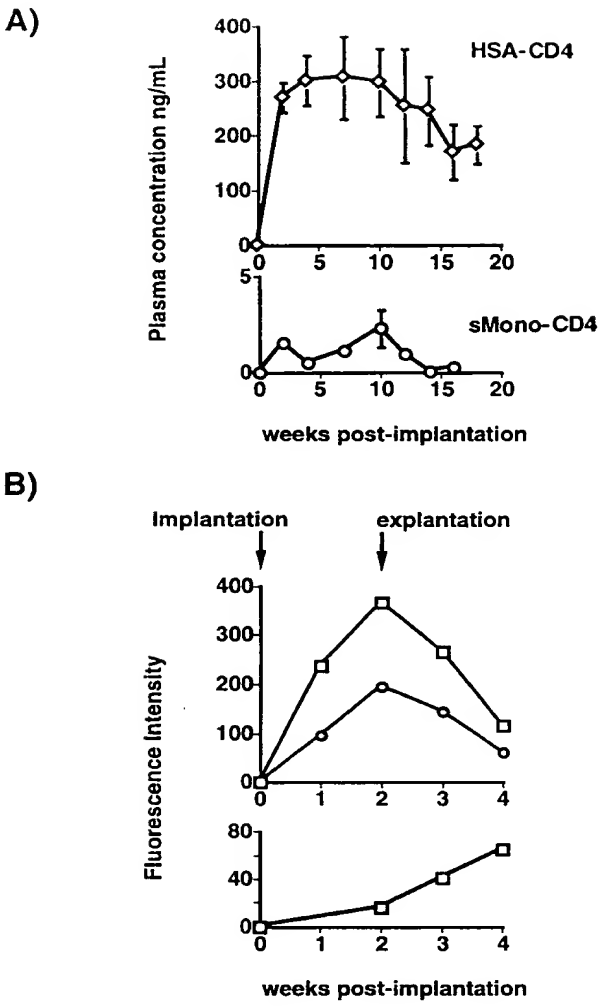

Figure 4. A) In-vivo expression of HSA-CD4 and sMono-CD4 encapsulated in human serum albumin alginate-coated beads in nude mice: Swiss nude mice were implanted either with the encapsulated 293/CI/HSA-CD4 (upper panel) or 293/CI/sCD4 cells (lower panel), then bled at the indicated number of weeks after the implantation. The plasma concentration of those molecules was determined by ELISA and is presented as mean ± SEM. B) In-vivo expression of BL4 mAb in BALB/c mice: Upper panel: The encapsulated BL4 hybridoma cells were implanted in two BALB/c mice for two weeks, then the beads were explanted. During two weeks of implantation, fluorescence intensity continued to increase and started to decrease after their explantation. Lower panel: The beads were left implanted in a Balb/c mouse for 30 days. The fluorescence intensity continued to increase for 30 days.

Transplantation in immunocompetent mice: in-vivo secretion of BL4 anti-CD4 mAb from the encapsulated BL4-hybridoma cells in Balb/c mice

Sera from BALB/C mice implanted with the BL4-hybridoma cells containing human serum albumin alginate beads were collected at various times after the implantation and analysed. The results showed that serum BL4 anti-CD4 mAb concentration continuously increased during the 30 days of follow-up when the beads were left in place *(figure 4B, lower panel)*. In contrast, when the beads were explanted after two weeks, BL4 anti-CD4 mAb secretion gradually decreased *(figure 4B, upper panel)*.

Mice were sacrificed, then the beads were recovered, put in culture medium in vitro, and subsequent BL4 anti-CD4 mAb secretion was verified (data not shown).

Histologic analysis of the beads implanted in mice

Beads harbouring either allogeneic or xenogeneic cells were explanted from either nude or immunocompetent mice and analysed. 293/sCD4- or 293/HSA-CD4-containing beads recovered from nude mice 16 weeks after implantation remained intact *(figure 5A)*, with fibrosis at the surface *(figure 5B)*, and viable cells clustered at the center of the beads *(figure 5C)*.

The 293/sCD4-containing beads explanted from immunocompetent BALB/c mice were still closed *(figure 5D)* but their outer membrane was altered by an extensive immune response from the host, with many lymphocytes as well as phagocytes, which digested the surface of the membrane *(figure 5E)*. The 293 cells appeared severely affected as well.

In contrast, the beads containing encapsulated BL4 hybridoma cells implanted in Balb/c mice remained intact at least until four weeks post-implantation *(figure 5F)*, surrounded by fibrosis and neo-vascularisation despite a wall originating from a xenogeneic human serum albumin *(figure 5G)*. The host immune response against the beads was moderate and BL4 hybridoma cells appeared healthy. Viable cells were seen at the center of the beads *(figure 5H)*.

DISCUSSION

Parameters affecting the plasma concentration of therapeutic protein secreted by genetically-modified cells

Many diseases may benefit from in-vivo delivery of therapeutic proteins by genetically-modified cells. The first question in regard to the feasibility of such approaches could be whether the therapeutic level of the protein is likely to be achieved with the methods envisioned. These levels are likely to depend on the secretion capacity and the number of the implanted genetically-modified cells, but they also depend on the pharmacokinetic properties of the protein. The treatment of HIV infection with CD4-based soluble inhibitors offers a good paradigm to study various strategies to produce these recombinant therapeutic proteins by implantation of genetically-modified cells. Indeed, it has long been shown that a soluble form of the HIV receptor (sMono-CD4) could efficiently neutralize culture-adapted HIV isolates in vitro. However, because of its quick in-vivo clearance (45 minutes in humans), the high dose of sMono-CD4 (3 to 10 mg) needs to be injected every eight hours in humans to maintain therapeutic concentrations [28]. This prompted the development of CD4-based recombinant molecules that were designed to have better pharmacokinetic properties, as well as improved efficacy. Such molecules are still under development as purified recombinant proteins to be injected into patients. They should be useful for obtaining high plasma concentration for a short period of time. However, for chronic diseases like HIV infection, another treatment modality such as the in-vivo delivery of CD4-derived protein by genetically-modified cells can be envisioned for a long-term delivery. Using stable 293 cells secreting three different CD4-based proteins with different half-lives, we generated organoids. We observed that plasma half-life is a major parameter that determines plasma concentrations. In addition, taking advantage of the fact that organoids made of 293 cells are tumourigenic in nude mice, we also showed that there was a linear relationship between the number of secreting cells and the plasma concentrations that rose up to 125 µg/mL with HSA-CD4. Thus, the number of secreting cells and the plasma half-life of the secreted recombinant protein are indeed two main parameters for obtaining high plasma concentrations.

sMulti-CD4, a potentially clinically useful inhibitor of HIV infection

Soluble CD4 (sMono-CD4) has been shown to neutralize a wide range of culture-adapted and primary HIV isolates in vitro [25, 29-34] by preventing a virus from binding to its receptor [35] , and also to inhibit cell-to-cell transmission. Clinical trials have shown that purified sMono-CD4 has no significant clinical or immunological toxicity, and induced a significant decline of the

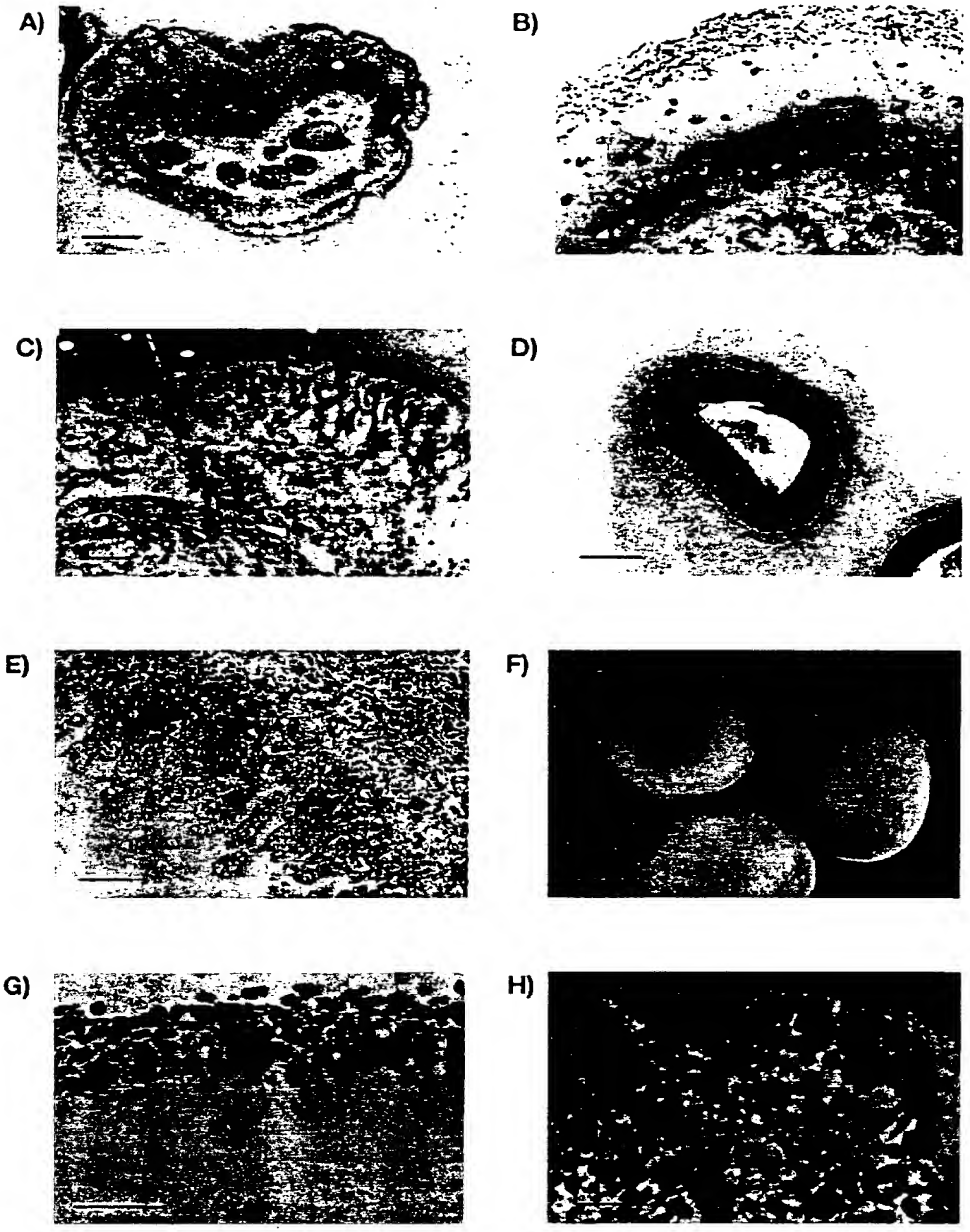

Figure 5. Encapsulated xenogeneic cells in nude mice. The coated alginate beads containing 293/CI/sCD4 cells recovered from a Swiss nude mouse 66 days post-implantation. **A, B)** No major degradation of the membrane by the host cells, but only the fibrosis was seen around the bead. C) In the center of the bead, clusters of the 293 cells alive were seen. Bars: A) 500 μm; B) 200 μm; C) 100 μm. Encapsulated xenogeneic cells in Balb/c mice. **D, E)** Human serum albumin alginate-coated beads containing 293/CI/sCD4 cells recovered from a Balb/c mouse one month after implantation. The membranes of the beads were strongly affected by the host phagocytes to develop fibrosis and vascularization around the beads. The 293 cells in beads show the morphological characteristics of dead cells. Bars: D) 1 mm; E) 100 μm. Xenogeneic beads in Balb/c mice. Human serum albumin alginate-coated beads containing BL4 hybridoma cells, removed from a Balb/c mouse 1 month after implantation. **F, G)** The membrane of the beads did not show any major deterioration and remained intact. Only slight fibrosis was seen around the beads. H) In the center of the beads, BL4 hybridoma cells were alive. Bar indicates in F) 1 mm, G) 100 μm and H) 50 μm, respectively.

viral load [16, 28]. However, because of its rapid in-vivo clearance (45 min–1 h in humans), it was necessary to inject a large dose of sMono-CD4 (3 to 10 mg) every eight hours to maintain plasma concentrations at therapeutic levels [28]. IgG-CD4 fusion protein has also been developed, which has a significantly prolonged plasma half-life [30]. It also appears to be more efficient at neutralizing HIV due to their di- or tetra-valence [17]. We have already shown that a minimal C4bp C-terminal α-fragment derived from a naturally heptameric molecule is sufficient to obtain soluble heptameric fusion protein [22]. Multimers are assembled in the cell without the necessity of secondary modifications, resulting in the secretion of a unique, covalently linked soluble molecule, although the expression vector codes only for the monomer. We used the same strategy to produce a soluble multimeric multivalent CD4, sMulti-CD4, in order to have a longer plasma half-life and a better antiviral activity than those of sMono-CD4. We indeed report here that sMulti-CD4 is approximately three times more efficient than sMono-CD4 at neutralizing HIV infection. In addition, although we have not determined it precisely, the plasma half-life of sMulti-CD4 should be significantly longer than that of sMono-CD4, being compatible with in-vivo use. Finally, sMulti-CD4 has a good physicochemical stability in vivo as shown by immunoblotting, as well as the antiviral effect of in vivo-expressed sMulti-CD4. Altogether, these results warrant further development of this molecule for HIV treatment, notably using gene therapy.

Serum albumin alginate beads coated with cross-linked bio-polymer as a tool for encapsulating genetically-modified cells

The use of an organoid to secrete recombinant protein suffers from some difficulties. Indeed, because genetically-modified cells are to be reimplanted into a recipient, they need to be autologous to prevent their rejection by the host immune responses. Therefore, they are usually derived from autologous skin fibroblasts, which need to be transduced and then expended before reimplantation. This is a labour-intensive and costly procedure which has to be tailored to every patient. The use of an 'universal' cell line expressing the protein of interest to be reimplanted in any patient would obviously greatly simplify the therapeutic procedures.

However, such allogeneic or xenogeneic cells need to be protected from the host immune response. Many encapsulation processes have been developed to achieve this goal [12, 14, 37, 38]. Here we have used human serum albumin alginate-coated beads to encapsulate human or mouse cells [23-36]. This method has the advantages of generating large beads that are quite solid, can be easily manipulated and contain significant numbers of cells. After encapsulation of genetically-modified 293 cells, we detected expected levels of sMono-CD4 and HSA-CD4, but only very low levels of sMulti-CD4, which is the product of the CD4-C4bpα gene. When we encapsulated the hybridoma cells secreting an IgG, the antibody was secreted from the beads. Therefore, the molecular cut-off for this type of bead should be between 150 and 350 kDa.

When the encapsulated cells were implanted in immunodeficient nude mice, xenogeneic cells survived and secreted the recombinant protein for a long period of time *(figures 5A, B, C)*. Interestingly, although 293 cells are tumourigenic in nude mice, no tumour could be detected, indicating that the coated alginate beads could protect the host from the tumourigenic cells within the beads.

When similar encapsulated cells were implanted in normal Balb/c mice, the xenogeneic cells (293 cells) were rapidly eliminated *(figures 5D, E)*, most probably due to an antibody-mediated immune response. However, when allogeneic cells (BL4) were reimplanted in immunocompetent animals (Balb/c mice), they survived and secreted the recombinant protein *(figures 5F, G, H)*. Altogether, these results demonstrate that the alginate-coated beads can protect allogeneic but not xenogeneic cells from immune rejection. If such results can be confirmed in primates, this should be useful for clinical use, which is likely to rely on allogeneic rather than xenogeneic cells.

Usually, therapeutic proteins need to be delivered under strict control to avoid overexpression and possible side effects. There are several promoters available which could be controlled in vivo, such as a system using tetracycline [39] or rapamycin [40]. Efficient long-term gene transfer into muscle tissue for immunocompetent mice by adeno-associated virus (AAV) vector has been reported [41-46]. It is also reported that control of gene expression in vivo after gene transfer using the AAV vector is possible [6, 47]. Although AAV is still difficult to produce under good manufacturing practice (GMP), this may represent a more practical method than the use of encapsulated cells. However, the latter method has two potential advantages: first, it does not require injecting a recombinant viral vector that always carries the risk of dissemination; second, implanted beads can always be explanted to stop recombinant protein production if necessary. This might provide an important safety control

when the overexpression of a therapeutic protein can trigger severe side effects.

In summary, allogeneic cells encapsulated in calcium-alginate beads coated with cross-linked bio-polymers appear a promising system for the in-vivo delivery of therapeutic proteins, including immunoglobulins.

ACKNOWLEDGEMENT

We thank Delphine Bohl (Institut Pasteur, Paris, France) for sharing her expertise in organoid preparation and implantation. We are grateful to Catherine Durieu and Eric Jeanpierre for their excellent technical assistance.

E.S. is a recipient of a postdoctoral fellowship from the ANRS.

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